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(54) Title: RECEPTOR DIRECTED-TOXIN CONJUGATES

(57) Abstract

The invention relates to a targeted toxin molecule comprising a toxin and a proteinaceaous molecule with binding activity for a receptor on the surface of a target cell, whereby the proteinaceous molecule is the ligand on a functional derivative or fragment thereof and the toxin has a molecular weight of no more than 1500 D. More specifically the ligand is IL-2, or preferably only its binding domain. The toxin may be any small toxin, for instance calicheamycin, verrucarin etc.. The toxin molecules according to the invention localize faster and are less immunogenic.

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Receptor directed-toxin conjugates.

The present invention relates to targeted toxin molecules comprising a toxin and a (proteinaceous) molecule with binding activity for a receptor on the surface of a target cell.

If the targeted toxin molecules are directed to a receptor which is specific for a certain group of cells, these cells can be specifically eliminated.

Such targeted toxin molecules are useful in tumortherapy whereby the receptor on the cell surface is specific for tumor cells or at least is preferentially expressed by said tumorcells.

The targeted toxin molecules can also be used in preventing allograft rejection and/or autoimmune diseases. In both the latter cases the aim is to suppress the cells (lymphocytes) which are responsible for the allograft rejection or the autoimmune response.

Early attempts to suppress the lymphocytes employed antisera directed against the entire lymphocyte population of a patient. These antisera have been shown to be useful in for instance kidney transplantations (1, 2), in spite of the fact that the patient is temporarily immuno-incompetent.

Later attempts employ antisera directed against a specific subpopulation, e.g. T-helper cells. These antisera also proved very helpful in combating both human and animal autoimmune diseases and allograft rejections (3, 4, 5, 6). But still, an entire subpopulation of lymphocytes is suppressed, resulting in a temporary partial immuno-incompetence of the

patient. Therefore, expensive protective precautions are still necessary.

A second disadvantage of methods using antisera is that the antisera usually are of animal origin. Eventually they will evoke an immunoresponse by the patient which will limit the use of such antisera to a very restricted number of administrations.

Therefore, more specific and less antigenic approaches are still desired.

An elegant approach to selectively suppress or eliminate a certain subset of cells is to direct a conjugate of a toxin, or possibly a radionuclide, and a (usually) proteinaceous substance with binding specificity for the receptors on the surface of said subset of cells to that subset, making use of the binding of said proteinaceous substance to the cellsurface.

Though most ligands will be of a proteinaceous nature, other compounds with a specificity for a certain subset of cells are also considered to be within the scope of this invention.

Two ways of targeting the toxin to receptors are known. One method employs a (monoclonal) antibody or a fragment thereof, which is directed to the receptor.

The other way is to make use of the natural ligand (or an agonist or antagonist) for the receptor or a functional fragment or derivative thereof, which can be coupled to the toxin. Also, agents which mimick the ligand may be used. Thus, in this specification the expression ligand is intended to also include fragments,derivatives or mimicking agents of either proteinaceous or other nature.

A subset of cells of particular interest for the above-mentioned methods of suppression or elimination is the set of activated T-cells of a patient with an unwanted immunoresponse.

It is well known that during the activation processes leading to an (auto-) immunereaction, T-cells express high numbers of Interleukin-2 receptors. These receptors bind Interleukin-2 (IL-2) that may also be produced by these cells (Autocrine stimulation). Consequently, the cells start to proliferate.

Again, two ways exist to target toxins to the IL-2 receptor: through IL-2 or fragments or derivatives thereof and through antibodies directed against IL-2 receptors.

The approach via monoclonal antibodies or fragments thereof has been described in literature (7, 8, 9 ,10, 11) and proven its efficacy both in vitro and in vivo in animals, in malignancies in humans and in rheumatoid arthritis.

The approach through monoclonal antibodies has the earlier mentioned disadvantage that the antibodies (usually of murine origin) evoke an immunoresponse by the patient. This may result in a diminished response, sensitisation and anaphylactic shock (12).

The targeting to the IL-2 receptor via its natural ligand conjugated to a toxin was recently reported (13).

It was shown in in vivo and in vitro studies that the antigen-activated circuit could be eliminated by administering Pseudomonas exotoxin conjugate. Also shown was that the IL-2-toxin only binds to the high affinity IL-2 receptor.

However, the Pseudomonas exotoxin is a toxin with a high molecular weight which will, as antibodies and/or antigens of foreign origin do, elicit an immunoresponse in the patient.

The present invention provides a toxin which will not evoke such an immuneresponse.

The present invention provides a targeted toxin molecule comprising the ligand or a functional

fragment or a derivative of one of both, for a receptor on the cell surface of a target cell and a toxin with a molecular weight of no more than 1500 D.

When the ligand chosen has binding activity for the IL-2 receptor the targeted toxin molecules of the invention are particularly suitable as therapeutics for autoimmune diseases in general and for rheumatoid arthritis in particular.

Furthermore the targeted toxin molecules may be used to remove IL-2 expressing haematopoietic cell leukemias, to induce specific tolerance in transplantation patients or in patients receiving treatment with an antibody of foreign origin.

In order to even further reduce the antigenicity of the targeted toxin molecules of the invention fragments of the ligand specific for the chosen receptor may be used.

These fragments preferably should comprise (an amino acid sequence of) the receptor binding domains of the ligand or a functional derivative thereof.

In the case of IL-2, the receptor binding domains appear to lie within the fragments comprising the 33-56 and the 11-20 amino acid sequences of IL-2.

The first fragment appears to bind the p55 chain of the IL-2 receptor, whereas the second fragment appears to bind the p75 chain. An additional advantage of these fragments is that they may be produced synthetically without time consuming genetic engineering and/or purification.

Instead of immediate targeting of the conjugates to a cell surface receptor, it is also possible and sometimes preferable to choose a pretargeting scheme.

A pretargeting scheme actually creates an "address" site (receptor) on the target molecule, which can be recognized by a conjugate according to the invention.

This is usually achieved by administering a first conjugate comprising apart which recognizes the target and a part which can be recognized by a conjugate according to the invention.

Pretargeting schemes have the advantage that there is no toxic compound present during localization of the first targeting moiety so that the damage to non target tissues can be reduced, especially when the specificity of the first targeting moiety is not too high.

Of course there are many other possible receptors to which the toxin molecule may be targeted. Interesting receptors are for instance lymphokine receptors, all T-cell receptors, Acetyl Cholin receptors, Epidermal Growth Factor receptors, Luteinizing Hormone receptors, Tumor Necrosis Factor α receptors, Transforming Growth Factor β receptors, reproductive hormone recptors (such as the hCG receptor) and so on.

Many suitable toxins for the targeted toxin molecules are readily available.

The important criterion in selecting the toxin is its lack of antigenicity, which is related to the molecular weight of the toxin. Suitable toxins will usually have a molecular weight of less than 1500 D, although this figure is no more than a guide-line. They may for instance be chosen from the following list:

diyn-ene- toxins like calicheamicins, esperamycins and dynemicin A, tricothecenes like verrucarin A, deoxyverrucarol, roridin A and diacetoxyscirpenol and mycotoxin. Especially preferred are calicheamicin and

verrucarin A. Of course it is also possible to use radionuclides as cytotoxic moieties.

The toxins and (proteinaceous) molecules according to the invention may be coupled to each other in any suitable way. Both the (proteinaceous) molecules and the toxins have sufficient reactive groups so as to introduce reactive groups that they may be coupled without a substantial detrimental effect to their respective activities. Alternatively the linkage between the toxin and targeting moiety may be broken upon localization or internalization of the conjugate.

The coupling may be either direct or through a linking molecule and/or a spacer.

The invention also relates to pharmaceutical compositions comprising the targeted toxin molecules according to the invention.

Obvious routes of administration for the compounds of the invention would be intra-articular or parenteral administration whereby the targeted toxin molecules are dissolved or emulsified in a suitable vehicle for injection. Apart from this vehicle the composition may of course contain the other usual additives.

The therapeutic dose of the conjugates according to the invention will vary with the molecular weight of the conjugates. It may range from 10 μg to 500 mg per injection in systemic applications. In local applications it may be even lower than that.

The invention will be described in more detail in the following examples.

Examples

1.1 Verrucarin A-2'-Succinate (1)

To a solution of Verrucarin A (200 mg, 0.4 mmol) in DMF (2.5 ml) were successively added Succinic Anhydride (85 mg, 0.8 mmol) and 4-Dimethylaminopyridine (4.8 mg 0.04 mmol). The mixture was stirred for 16 hr. at 50 °C. The reactionmixture was diluted with CH_2Cl_2 (dichloromethane) (8 ml) and washed with 5 % aq. Citric Acid (2 x 8 ml) and water (8 ml). The organic layer was separated and the solvent was removed by evaporation under reduced pressure to yield crude 1. Verrucarin A-2'-Succinate was purified by chromatography on silica (eluent: CH_2Cl_2/CH_3OH = 98/2) to yield 1 (148 mg, 61.5 %). TLC R_f(A) 0.35.

1.2. N-Succinimidyl-Verrucarin A-2'-Succinate (2)

To a cold (0°C) solution of Verrucarin A-2'-Succinate (148 mg, 0.24 mmol) in CH_2Cl_2 (Dichloromethane) (2 ml) were successively added EDCI (N-ethyl, N'-3-dimethylamino-propylcarbodiimide) (56.3 mg, 0.29 mmol) and HONSu (N-hydroxy-succinimide) (33.8 mg, 0.29 mmol). The reaction mixture was stirred at 0°C for 15 min. and then kept at room temperature for 2 hr.
The reaction mixture was washed successively with 5 % aq. citric acid (4 ml), water (4 ml) and sat.aq. NaCl (4 ml), dried over sodium sulphate and concentrated in vacuo yielding 188 mg of 2 (100 %). TLC R_f(A) 0.60.

2.1. Verrucarin A-2'-Hemisuccinoylhydrazide (3)

2(60 mg, 0.086 mmol) was dissolved in tetrahydrofuran (1 ml). 5 eq. Hydrazine hydrate in methanol were added and the reaction mixture was stirred at room temperature for 15 min. The reaction mixture was diluted with dichloromethane (4 ml) and washed successively with 5 % aq. $KHSO_4$ (4 ml), sat. aq. NACl (4 ml), dried over sodiumsulphate and concentrated in vacuo. The resulting crude hydrazide was purified by chromatography on silica (eluent: CH_2Cl_2/CH_3OH = 95/5) to yield 3 (30 mg, 56.8 %)

TLC R_f= 0.30. TLC :Dichloromethane/Methanol = 95/5

3.1. Spectroscopic data for VA-derivatives ('H NMR)

Proton	Verrucarin A	1	2	3
2	3.86 d	3.86 d	3.86 d	3.86 d
3	2.23/2.50 m	2.23/2.50 m	2.23/2.50 m	2.23/2.50 m
4	5.81 dd	5.80 dd	5.80 dd	5.80 dd
7	1.90 m	1.90 m	1.90 m	1.90 m
8	1.90 m	1.90 m	1.90 m	1.90 m
10	5.40 dd	5.40 d	5.40 d	5.40 d
11	3.55 d	3.56 d	3.56 d	3.57 d
13	2.80/3.12 (AB)	2.80/3.12	2.80/3.12	2.80/3.12
14	0.84 s	0.85 s	0.86 s	0.85 s
15	4.20/4.80 (AB)	4.20/4.80	4.18/4.68	4.19/4.68
16	1.75 s	1.72 s	1.74 s	1.75 s
2'	4.14 s	4.84 d	4.87 d	4.84 d
3'	2.35 m	2.48 m	2.48 m	2.50 m
4'	1.90 m	1.90 m	1.90 m	1.90 m
5'	4.02/4.52	4.02/4.50	4.02/4.47	4.00/4.48
7'	6.05 d	6.05 d	6.05 d	6.05 d
8'	8.03 dd	8.00 dd	8.00 dd	8.00 dd
9'	6.67 t	6.67 t	6.67 t	6.67 t
10'	6.13 d	6.13 d	6.13 d	6.13 d
12'	0.87 d	1.02 d	1.04 d	1.02 d
1''		2.71 s	2.85 s	2.85 s
2''		2.71 s	2.85 s	2.50 t
3''			2.85 s	
4''			2.85 s	
NH-Hydrazide				6.98 s

4. Procedures.

4.1. The conjugates were made by adding VAONSu (1mg/ml, DMF) to the protein (0.1 M Sodiumphosphate pH 8.5: DMF = 2 : 1) in a ratio 10 : 1 (mol/mol). After 10 min. incubation the conjugates were purified on a PD 10 column, equilibrated in PBS buffer. This was done for the proteins Interleukin-2 (IL-2), Transferrin, hCG and Epidermal Growth Factor (EGF). The peptide-VAONSu conjugates were made by adding VAONSu (1 mg/ml, DMF) to the peptide (dissolved in 0.1 M Sodiumphosphate pH 8.5: DMF = 2 : 1) in a ratio 1 : 1 (mol/mol). After an incubation period of 10 min. the peptide conjugates were purified by adding a 20 fold excess ethyl-acetate (vol/vol). After centrifugation, the precipitate was dissolved in PBS buffer. This was done for the p20 peptide, the internalizing part of (IL-2) and the J6-peptide.
Verrucarin -A-hydrazide was linked to carbohydrate groups on Transferrin.
The carbohydrate groups were oxidised by adding 10 mM NaIO4 to Transferrin, dissolved in 0.1 M NaOAc pH 5.5. After 10 min. Na_2SO3 was added in order to remove excess NaIO4. Then V-A-hydrazide was added (concentration 1 mM). After 1 hour incubation the conjugate was purified on a PD 10 column, equilibrated in PBS buffer.

4.2 Tests.

4.2.1 A human T cell clone (Reiz 1F9) was cultured together with the various Verrucarin A conjugates in M505 medium supplemented with 10 % human pool serum, 2 mM glutamine, 20 μM β-mercaptoethanol and antibiotics (200 U/ml penicillin, 100 mg/ml streptomycin). After 2 h. of incubation the conjugates were removed by washing the cells with medium.
Cells were then cultured in the presence of 20 U recombinant IL-2 at 37 C, in a humid 5 % CO_2 atmosphere for 72 hr. 3H-Thymidine (0.1 μCi/well) was then added and after an overnight incubation the cells were harvested and the incorporation of radioactivity was counted with a Packard beta counter.

4.2.2 PANC cells were grown in M505 + 10 % FCS. For testing, the PANC cells were cultured in microtiter plates (1000 cells/well, 100 uL). At day 0, 100 μl samples of ligand-toxin were added and cells were incubated in a 5 % humid CO_2 atmosphere. At day 8, 50 μl of MTT were added in order to measure cell survival. After 4 h. of incubation, medium was removed and the violet coloured crystals formed inside the cell were dissolved in 75 μl of DMSO.
The absorbance at 540 nm was read in an ELISA reader.

4.2.3 Leydig cells were isolated from the testes of mature Swiss mice (9 to 13 weeks old). The cells were obtained by sucking each decapsulated testis 5 times through a glass tube and filtering the suspension through a 30 µm nylon mesh. The cells were suspended in M199 supplemented with 4.2 mM $NaHCO_3$, 20 ml/l fetal calf serum and 1 g/l BSA and 100 µl cell suspension was added to each well of a microtiter plate along with 50 µl test sample. Plates were incubated for 4 h at 37 C in a humid atmosphere of 5 % CO_2-95 % air subsequently stored at -20°C until testosterone determination by RIA.

5 Results

5.1 In order to demonstrate the effectiveness, we have prepared and tested various ligand -verrucarin-A conjugates: viz linked to P20 (the binding part of IL-2 (AA 8-27) to the internalizing component of the IL-2 receptor: p75), J-6 peptide activating T cell clone in concentration 1,5 µM), transferrin, hCG and epidermal growth factor. In all experiments, it is shown that Verrucarin-A-ONSU ester retains its toxicity, indicating that the VA -derivative is still able to enter and kill the cells. It is well known that if Verrucarin-A is bound to a protein e.g. antibodies, the conjugate cannot enter the cell anymore and is, therefore, devoid of toxicity.
Various experiments were conducted using the T-cell clone . Fig. 3 shows that the P20-VA conjugate reduces the proliferation of the T

cell clone at relatively high concentrations, when compared to VA alone; P20 itself has no effects, indicating the specific toxicity of the conjugate; the binding affinity of P20 to the IL-2 receptor is relatively low, explaining the relatively high concentrations required for the toxic effects.
Fig. 4 shows that the J-6-VA conjugates kills the cell clone at very low concentrations (0.02-0,05μM), indicating very specific and efficient killing. At very high concentrarions the J-6 peptide itself generates some well known cytotoxic effects.

Using the PANC tumor cells the following results were obtained.
Again the VA-ONSU ester retains full toxicity of VA alone. If VA is conjugated to epidermal growth factor (fig. 5) or transferrin (fig. 6-7) specific inhibition of the growth of the PANC cells is found. The ligands themselves have no effects.

In yet another model system we tested the hCG-VA conjugates. After 4 h of incubation, hCG clearly stimulates - as expected - the testosterone production in mouse Leydig cells to the relative amount of about 600 ng/ml. Incubation with hCG-VA (1 mg/ml) significantly reduced the testosterone production in mouse Leydig cells.
The results of the incubation with hCG-VA in this test procedure are shown in fig. 8.

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List of Abbreviations.

IL-2	Interleukin-2
p 55	Subunit of the Interleukin-2 receptor
p 75	Subunit of the Interleukin-2 receptor
D	Dalton
DMF	DiMethylFormamide
TLC	Thin Layer Chromatography
NMR	Nuclear Magnetic Resonance
VA	Verrucarin A
ONSu	Succinimidyl ester derivative
PBS	Phosphate Buffered Saline
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
hCG	human Chorionic Gonadotropin
RIA	Radio Immuno Assay

Claims

1. A targeted toxin molecule comprising a toxin and a targeting molecule with binding activity for a receptor on the surface of a target cell, characterized in that said targeting molecule is the ligand or a functional derivative or fragment thereof for said receptor and that the toxin has a molecular weight of no more than 1500 D.

2. A targeted toxin molecule according to claim 1 or 2, characterized in that the targeting molecule is of a proteinaceous nature.

3. A targeted toxin molecule according to claim 1 or 2, characterized in that the targeting molecule has binding specificity for a lymphokine-receptor.

4. A targeted toxin molecule according to claim 1,2 or 3, characterized in that the targeting molecule has binding specificity for an acetylcholin receptor.

5. A targeted toxin molecule according to claim 1,2 or 3, characterized in that it has binding specificity for a reproductice hormone receptor.

6. A targeted toxin molecule according to claim 1, 2 or 3, characterized in that it has binding specificity for a TNF-α-receptor.

7. A targeted toxin molecule according to claim 1, 2 or 3, characterized in that it has binding specificity for a TGF-β-receptor.

8. A targeted toxin molecule according to claim 1, 2 or 3, characterized in that it has binding specificity

for an Interleukin-2-receptor.

9. A targeted toxin molecule according to claim 2, 3 or 8, characterized in that the proteinaceous molecule comprises the amino acid sequence of the receptor binding domain of Interleukin-2.

10. A targeted toxin molecule according to claim 9, characterized in that the proteinaceous molecule comprises amino acids 33-56 of Interleukin-2.

11. A targeted toxin molecule according to claim 9, characterized in that the proteinaceous molecule comprises the amino acids 11-20 of interleukin-2.

12. A targeted toxin molecule according to claim 1,2 or 3, characterized in that it recogrizes a T-cell receptor.

13. A targeted toxin molecule according to any one of the aforegoing claims characterized in that the toxin is chosen from diyn-ene- toxins like calicheamicins, esperamycins and dynemicin A, tricothecenes like verrucarin A, deoxyverrucarol, roridin A and diacetoxyscirpenol and mycotoxin.

14. A targeted toxin molecule according to claim 13, characterized in that the toxin is calicheamycin.

15. A targeted toxin molecule according to claim 13, characterized in that the toxin is verrucarin A.

16. A pharmaceutical composition comprising a targeted toxin molecule according to any one of the aforegoing claims and a suitable vehicle for administration.

FIG. 1

VERRUCARIN A

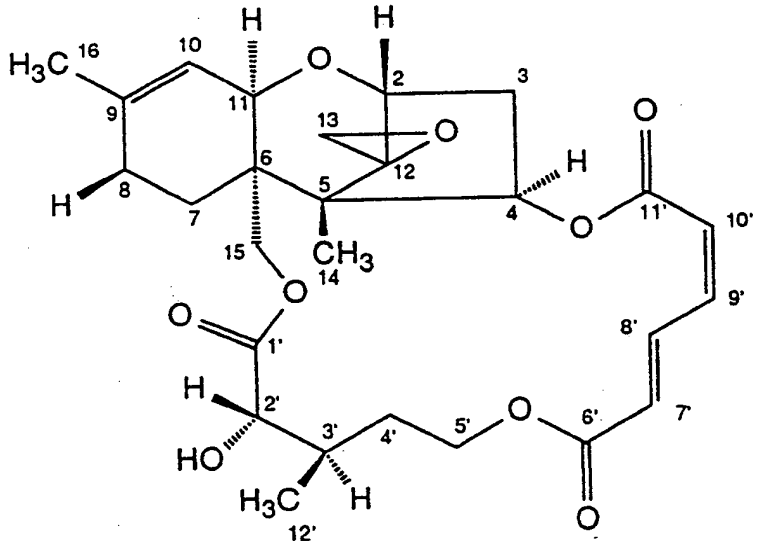


FIG. 2

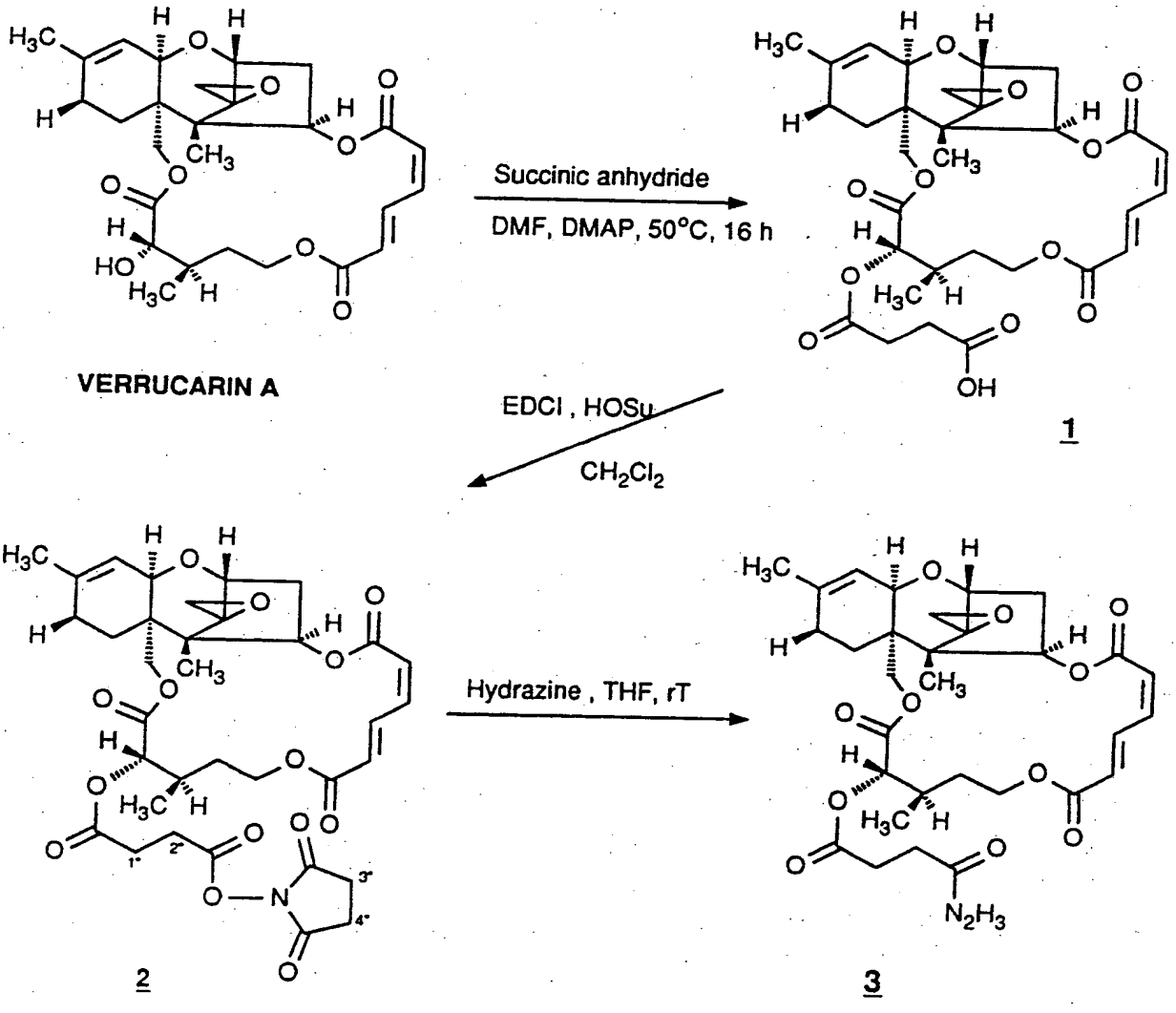

VERRUCARIN A
Succinic anhydride
DMF, DMAP, 50°C, 16 h
1
EDCI , HOSu
CH_2Cl_2
2
Hydrazine , THF, rT
3

FIG. 3

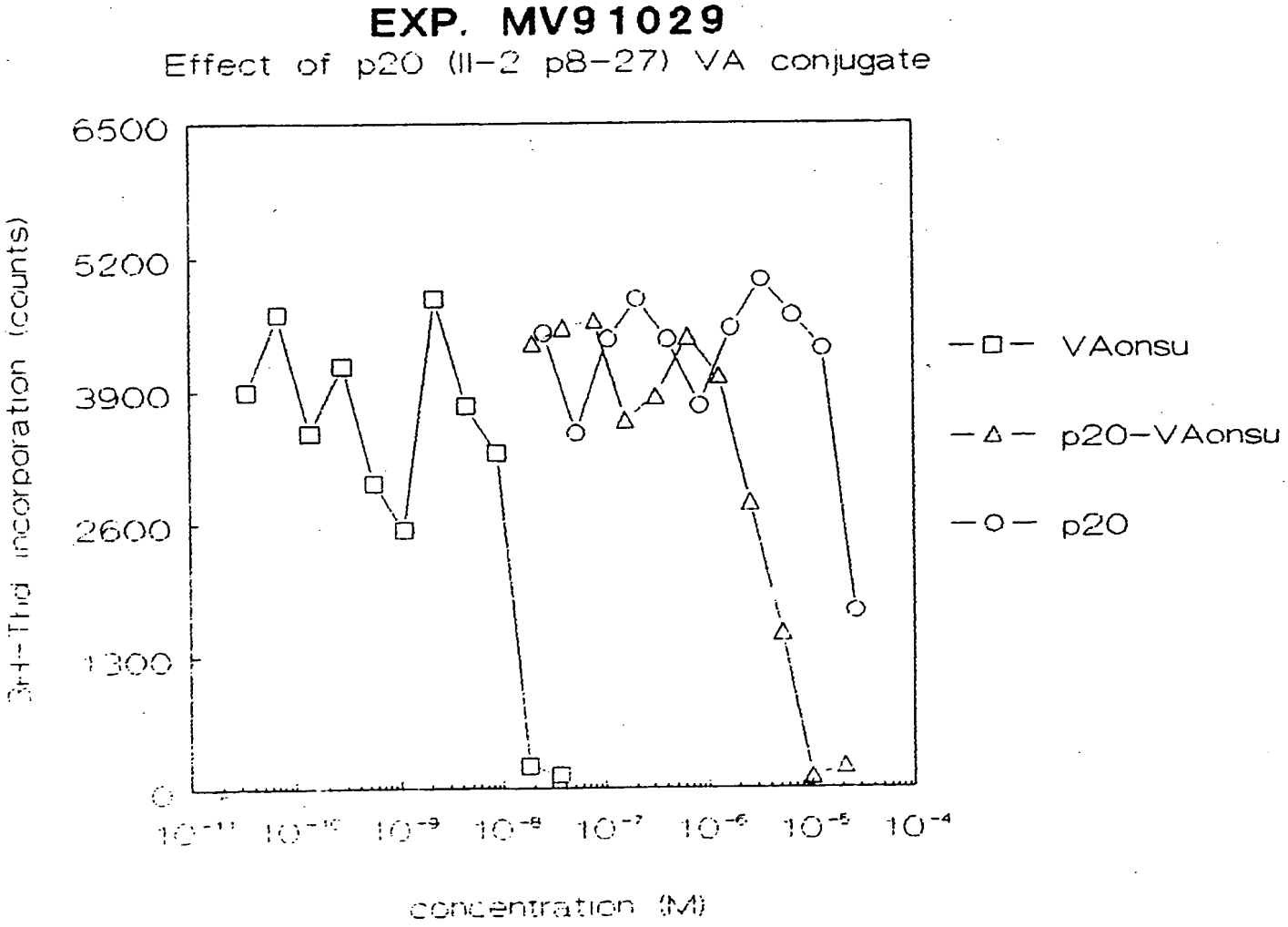

EXP. MV91029
Effect of p20 (Il-2 p8-27) VA conjugate
6500
5200
3900
2600
1300
0
3H-Thd incorporation (counts)
10^{-11} 10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4}
concentration (M)
VAonsu
p20-VAonsu
p20

FIG. 4

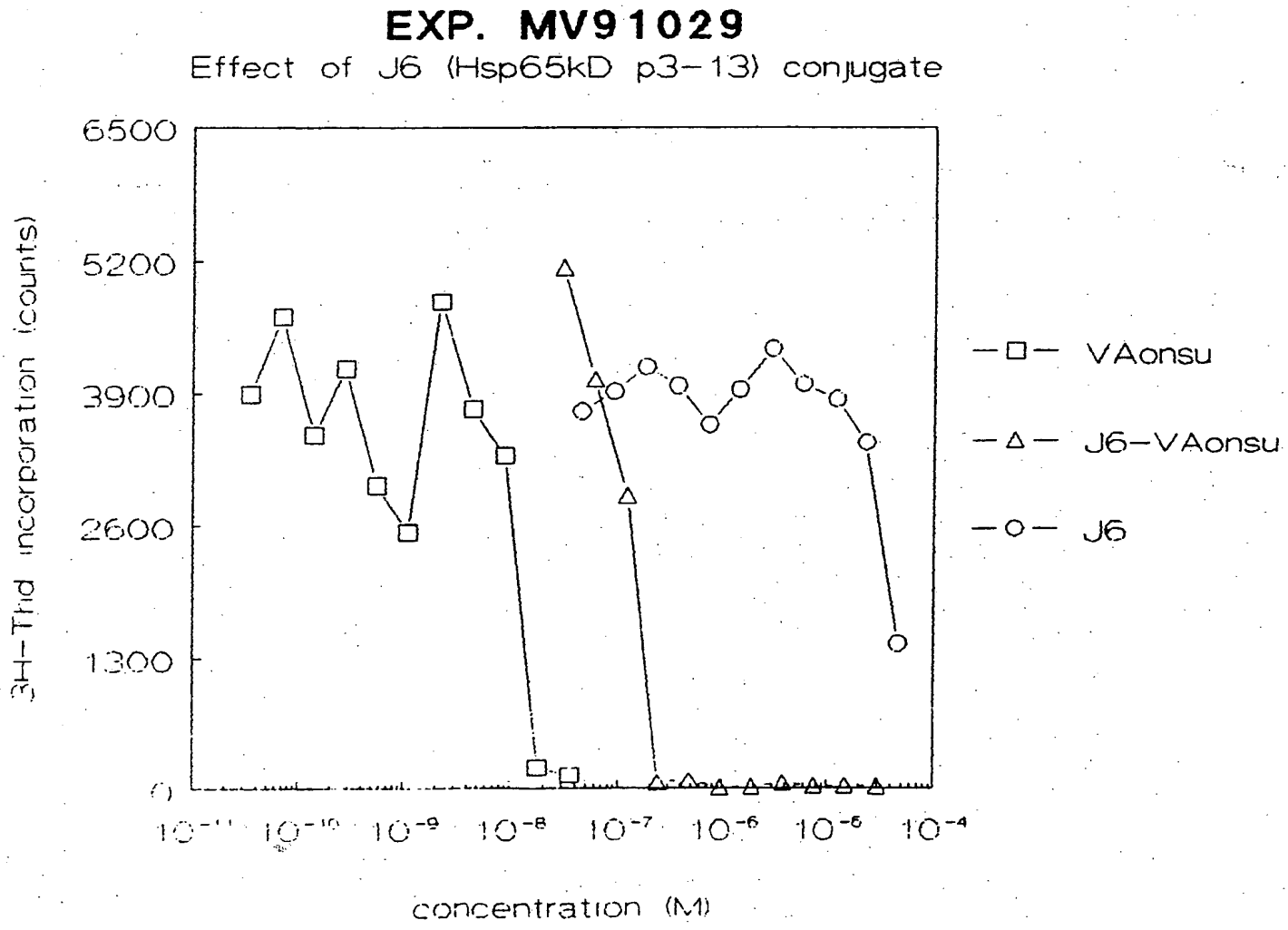

EXP. MV91029
Effect of J6 (Hsp65kD p3-13) conjugate
6500
5200
3900
2600
1300
0
3H-Thd incorporation (counts)
10-11 10-10 10-9 10-8 10-7 10-6 10-5 10-4
concentration (M)
VAonsu
J6-VAonsu
J6

FIG. 5

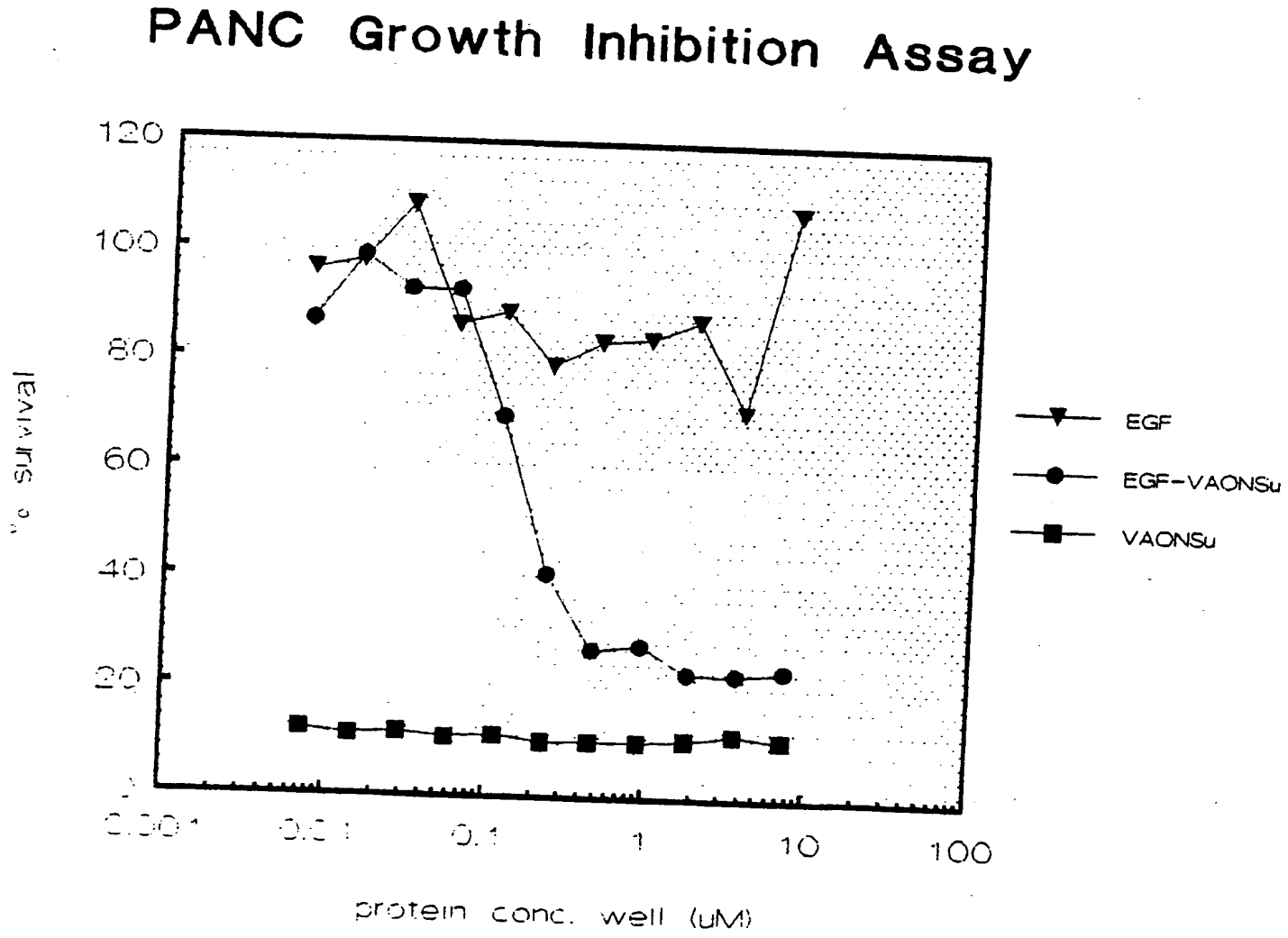

PANC Growth Inhibition Assay
120
100
80
60
40
20
0.001
0.01
0.1
1
10
100
% survival
protein conc. well (uM)
EGF
EGF-VAONSu
VAONSu

FIG. 6

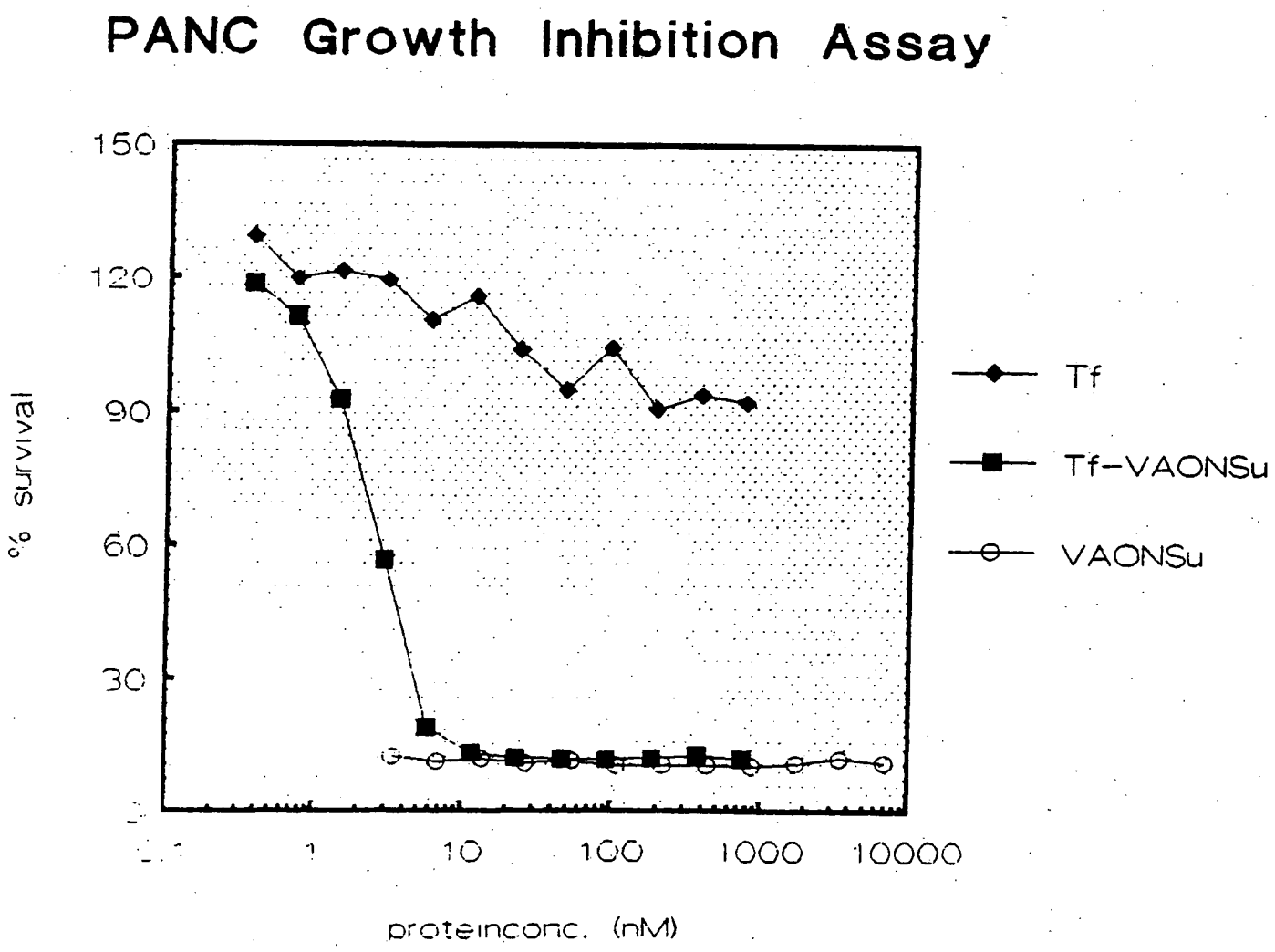

PANC Growth Inhibition Assay
150
120
90
60
30
% survival
1
10
100
1000
10000
proteinconc. (nM)
Tf
Tf-VAONSu
VAONSu

FIG. 7

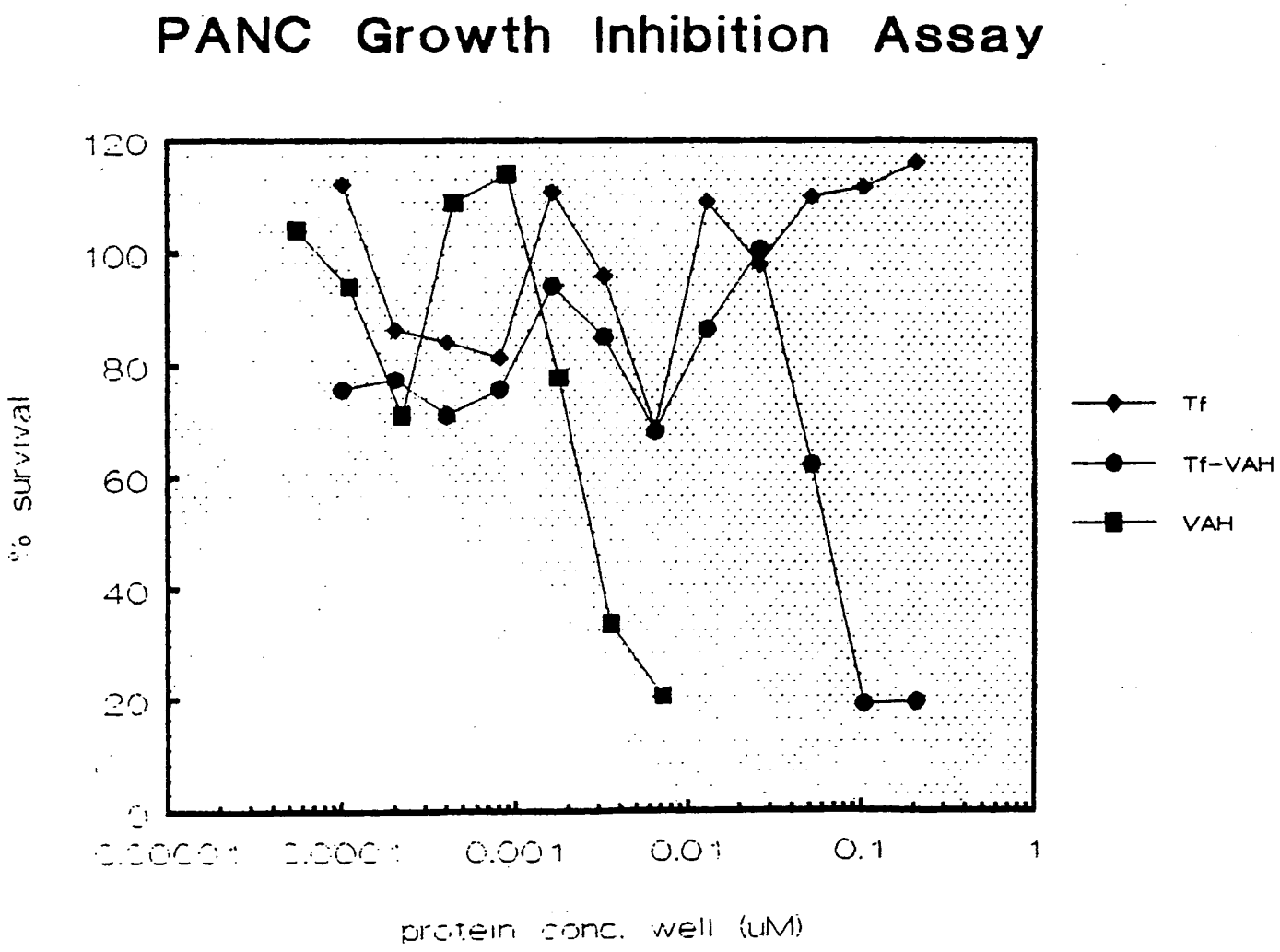
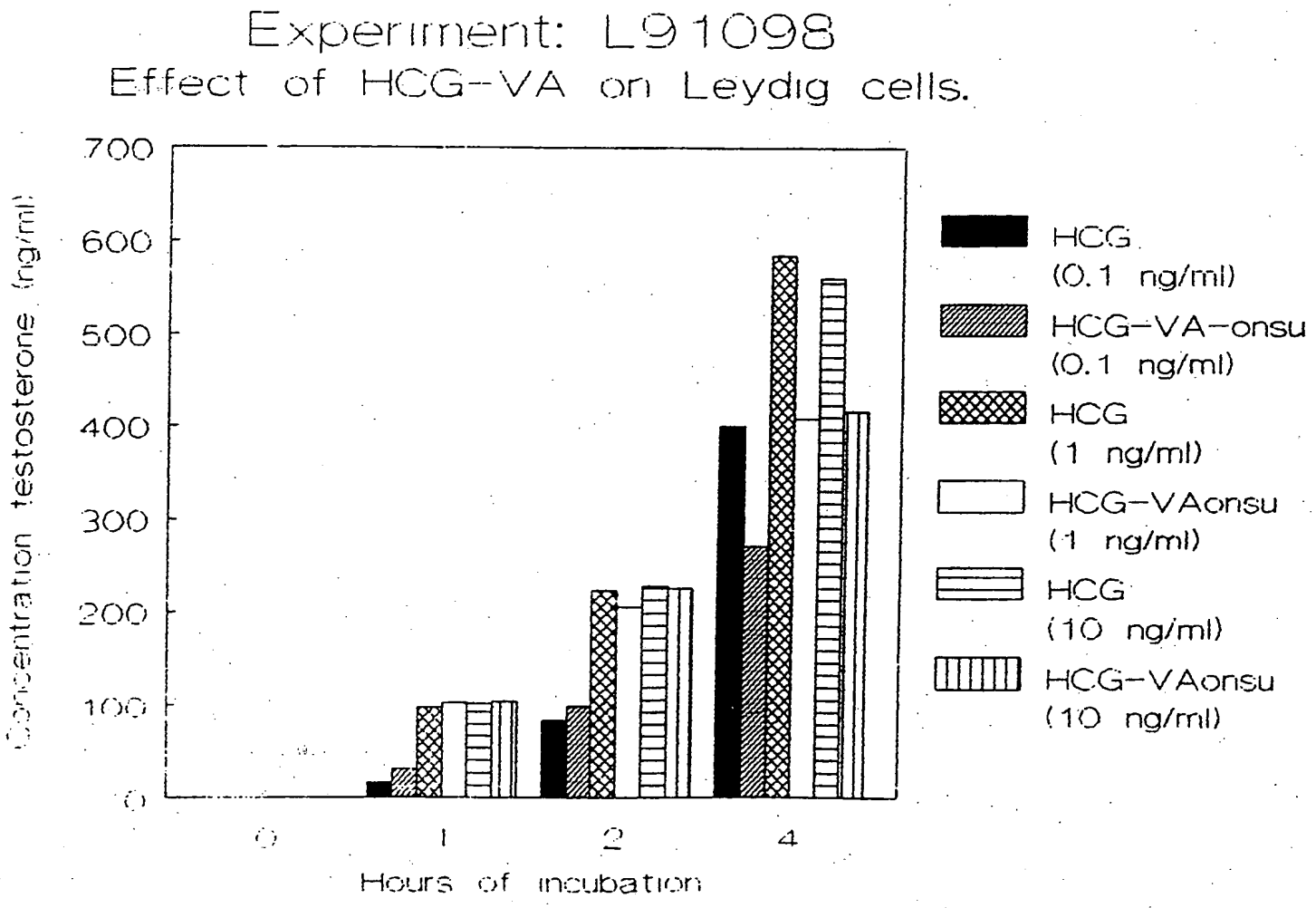

PANC Growth Inhibition Assay
% survival
120
100
80
60
40
20
0
0.00001
0.0001
0.001
0.01
0.1
1
protein conc. well (uM)
Tf
Tf-VAH
VAH

FIG. 8


Experiment: L91098
Effect of HCG-VA on Leydig cells.
Concentration testosterone (ng/ml)
700
600
500
400
300
200
100
0
0
1
2
4
Hours of incubation
HCG (0.1 ng/ml)
HCG-VA-onsu (0.1 ng/ml)
HCG (1 ng/ml)
HCG-VAonsu (1 ng/ml)
HCG (10 ng/ml)
HCG-VAonsu (10 ng/ml)

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 91/01169

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 A 61 K 47/48

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl.5	A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	WO,A,8911287 (THE BETH ISRAEL HOSPITAL ASSOCIATION) 30 November 1989, see pages 1-2; claim 5 ---	1-16
X	WO,A,9001951 (TANOX BIOSYSTEMS) 8 March 1990, see page 4, line 11; claim 11 ---	1-16
X	TRENDS IN BIOTECHNOLOGY, vol. 7, no. 3, March 1989, pages 57-61, (Cambridge, GB), M.L. FIANI et al.: "Selective targeting of drugs", see page 59, column 2, lines 4-9 ---	1-2
X	EP,A,0259904 (BATTELLE MEMORIAL INSTITUTE), 16 March 1988, see pages 3-5 ---	1-2
X	WO,A,9000565 (AMGEN) 25 January 1990, see page 25, table 1; claim 43 --- -/-	8-11

° Special categories of cited documents : [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
06-08-1991	24. 09. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. Pas

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8702061 (BIOTECHNOLOGY RESEARCH PARTNERS) 9 April 1987, see the whole document ---	1-16
A	EP,A,0319012 (E.I. DU PONT DE NEMOURS) 7 June 1989, see the whole document -----	1-16

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9101169 SA 48626

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/09/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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WO-A- 9001951	08-03-90	EP-A- 0429534	05-06-91
EP-A- 0259904	16-03-88	AU-B- 608531	11-04-91
		AU-A- 7788787	24-02-88
		WO-A- 8800837	11-02-88
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		JP-T- 3500415	31-01-91
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		EP-A- 0238645	30-09-87
EP-A- 0319012	07-06-89	AU-A- 2635088	08-06-89
		JP-A- 2000300	05-01-90